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(54) Title: HEPATITIS C SURROGATE VIRUS FOR TESTING THE ACTIVITY OF HEPATITIS C VIRUS PROTEASE, A RECOMBINANT GENE AND A USE THEREOF

(57) Abstract

The recombinant poliovirus which comprises poliovirus, hepatitis C virus (HCV) protease NS3 and target sites of the hepatitis C virus protease NS3, and needs the HCV protease activity, is produced, because efficient culture system for drugs against hepatitis C virus (HCV) which is the major etiologic agent of non-A, non-B hepatitis is lack.

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HEPATITIS C SURROGATE VIRUS FOR TESTING THE ACTIVITY

OF HEPATITIS C VIRUS PROTEASE, A RECOMBINANT GENE

AND A USE THEREOF

BACKGROUND OF THE INVENTION

5   **Field of the Invention**

The present invention relates to a hepatitis C virus (HCV), more

particularly, is related to a hepatitis C surrogate virus which comprises poliovirus

genome, HCV protease and its target site, and is suitable for testing efficacy of

anti-HCV drugs and screening the same, a recombinant gene and a use thereof.

10   **Description of the Related Arts**

There are known some types of viruses which cause viral hepatitis.

Hepatitis A virus is an RNA virus having a diameter of 27 nm and causing

epidemic hepatitis through oral infection.   Hepatitis B virus is a DNA virus having

a diameter of 42 nm and causing hepatitis through blood infection.   A hepatitis

15   virus which does not belong to any of the above types was called non-A, non-B

hepatitis virus.

Hepatitis C virus (HCV) is the major etiologic agent of non-A, non-B

hepatitis (Alter et al., N. Engl. J. Med. 321, 1494-1500, 1989; Choo et al., Science,

244, 359-362, 1989; Kuo et al., Science 244, 362-364, 1989).   This virus has

20   been implicated in liver cirrhosis and hepatocellular carcinoma (Bruix et al., Lancet

ii, 1004-1006, 1989; Saito et al., Proc. Natl. Acad. Sci. USA, 87, 6547-6549, 1990).

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At present, α-interferon is widely used for treating HCV patients. However, only about half of the patients respond to α-interferon, and about half of the responders suffer from a recurrence of the virus (Hino et al., C. J. Med. Virol., 42, 299-305, 1994; Tsubota et al., Hepatology, 19, 1088-1094, 1994). Development of other anti-HCV drugs is, therefore, necessary.

In order to develop the anti-HCV drugs, it is essential to set up HCV cultivation system to test the efficacy of the drugs. However, in vitro cultivation systems available to date are inefficient and technically difficult to manipulate (Yoo et al., J. Virol., 69, 32-38, 1995; Beach et al., Viral Hepatitis Research Foundation of Japan, 286, 67, 1993; Shimizu et al., Proc. Natl. Acad. Sci. USA, 89, 5477-5481, 1992). Furthermore, HCV titer is very low even in sera of hepatitis patients due to its nature of low yield. Accordingly, it is very difficult to screen or test for efficacy of antiviral drugs using HCV cultivation. Therefore, the surrogate virus will be very useful for studying HCV genes in virus replication and testing antiviral efficacy of drugs affecting HCV-encoded enzymes. Namely, the development of the surrogate virus for anti-HCV drugs is urgent.

SUMMARY OF THE INVENTION

Accordingly, the present invention is intended to overcome the above-mentioned disadvantage in the development of anti-HCV drugs and provide proper surrogate viruses for HCV suitable for the investigation of antiviral efficacy of drugs and proliferable in the HCV cultivation system.

An embodiment of the present invention is a recombinant gene coding hepatitis C surrogate virus comprising a virus gene coding picornavirus, a

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protease gene coding hepatitis C virus protease NS3 which is in an open reading

frame (ORF) of said picornavirus, and a target gene coding target sites of said

hepatitis C virus protease NS3 which is in said open reading frame of said

picornavirus.

5   Among the picornaviruses, the poliovirus is preferred.

The target site is preferred to be selected from the group consisting of

NS5A/5B, NS4A/4B and NS4B/5A.

The hepatitis C surrogate virus protease NS3 is preferred to cleave the

peptide bond between cysteine of P1 and glycine of P1' as a target site.

10   In another aspect, the present invention provides a screening method of

anti hepatitis C virus drugs comprising the step of detecting a material which

inhibits surrogate virus proliferation expressed by the recombinant gene for coding

hepatitis C surrogate virus.

In another aspect, the present invention provides a measuring method of

15   anti-hepatitis C virus drugs activity comprising the steps of proliferating the

surrogate virus expressed by the recombinant gene coding hepatitis C surrogate

virus in the presence of anti-hepatitis C virus agents, and measuring the

proliferating ability of the hepatitis C surrogate virus.

The present invention, in another aspect, provides a hepatitis C surrogate

20   virus comprising the recombinant gene for coding hepatitis C surrogate virus.

The poliovirus's versatility is used in developing surrogate virus suitable

for investigation of HCV protease activity.

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HCV belongs to the family *Flaviviridae* whose members are enveloped

viruses having a positive-sense RNA genome (Francki *et al.*, *Arch. Virol* 2(Sullo.),

223, 1991; Inchauspe *et al.*, *Proc. Natl. Acad. Sci.*, 88, 10292-10296, 1991; Miller

et al., *Proc. Natl. Acad. Sci.*, 87, 2957-2061, 1990; Takamizawa *et al.*, *J. Virol.* 65,

5   1105-1113, 1991).   The RNA encodes a polyprotein (-3010 amino acids) with the

following gene order: 5'-C'E1-E2-NS2-NS3-NS4A-NS4B-NS5A-NS5B-3'.   During

and/or after synthesis, the polyprotein is processed into functional proteins by

host- and virus-encoded proteases.   Core protein (C) and envelope proteins (E1

and E2) are believed to compose the structural elements of the virion particle.

10   The balance of the processed proteins is believed to function in replication

processes of the virus inside the host cells.   The signal peptidase on the

endoplasmic reticulum is responsible for the generation of the N-terminus of E1,

E2, and possibly NS2.   It has been suggested that the NS2/NS3 junction is

cleaved by an HCV-encoded metalloprotease, which resides between the C-

15   terminal portion of NS2 and the N-terminal region of NS3.   The cleavage of the

NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B sites depends on the activity of the

serine protease NS3 (Bortenschlager *et al.*, *J. Virol.* 68, 5045-5055, 1993; Eckart

et al., *Biochem, Biophys. Res. Commun.* 192, 399-406, 1993; Grakoui *et al.*, *J.*

Virol. 67, 2832-2843, 1993; Han *et al.*, *J. Gen. Virol.* 76, 985-993, 1995; Hijikata *et*

20   *al.*, *J. Virol.* 67, 4665-4675, 1993; Tomei *et al.*, *J. Virol.* 67, 4017-4026, 1993).

HCV protease NS3 is a good putative therapeutic target molecule for the

development of anti-HCV drugs, since its activity is considered essential for viral

proliferation.   Protease activity of NS3 resides in the N-terminal part of NS3

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(Bartenschlager et al., J. Virol. 67, 3835-3844, 1994; Han et al., J. Gen Virol. 76,

985-993, 1995; Lin et al., J. Virol. 68, 8147-8157, 1994), and NS4A enhances the

protease activity of NS3 (Bartenschlager et al., J. Virol. 68, 3835-3844, 1994;

Failla et al., J. Virol. 68, 3753-3760, 1994; Hahm et al., J. Virol. 69, 2534-2539,

5   1995; Lin et al., J. Virol. 69, 4373-4380, 1995; Tanji et al., J. Virol. 69, 1575-1581,

1995).   Characterization of the NS3 protease using known protease inhibitors has

revealed that NS3 is a chymotrypsin-like protease (Hahm et al., J. Virol. 69, 2534-

2539, 1995).

        The poliovirus is the prototype of picornaviruses.   The polioviral genome

10   is composed of a positive-sense RNA molecule that encodes a single open

reading frame (ORF) (Kitamura et al., Nature (London) 291, 547-553, 1981;

Racaniello et al., Proc. Natl. Acd. Sci. USA 78, 4887-4891, 1981).   Upon infection,

the genomic RNA is translated into a large precursor polyprotein via the internal

ribosomal entry site (IRES) in the 5'-nontraslated region of the RNA (Jang et al., J.

15   Virol. 62, 2636-2643, 1988; Pelletier et al., Nature (London) 334, 320-325, 1988;

Pelletiet et al., J. Virol. 63, 441-444, 1989).   The polyprotein, in turn, is processed

into the mature viral structural and nonstructural proteins by the 2A, 3C, and 3CD

proteases (Harris et al., Semin. Virol. 1, 323-333, 1991; Lawson and Semler., In

"Current Topics in Microbiology and Immunology" (V.R. Racaniello, Ed.), 161, 49-

20   87, 1990).   Poliovirus is one of the best studied viruses and is relatively easy to

manipulate and cultivate.   Production of infectious poliovirus from cDNA clones

has been practiced for a decade to study functions of poliovirus proteins (Molla et

al., Science 254, 1647-1651, 1991; Van der Werf et al., Proc. Natl. Acad. Sci.

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USA 83, 2330-2334, 1986) or for use of the poliovirus as a vector in delivering

foreign genes into host cells (Alexander *et al.*, *Proc. Natl. Acad. Sci.* USA 91,

1406-1410, 1994; Andino *et al.*, *Science* 265, 1448-1451, 1994; Lu *et al.*, *J. Virol.*

69, 4797-4806, 1995)

Inventors generated a hybrid poliovirus which requires the activity of HCV

protease NS3 for its proliferation, on the basis of the characteristics of HCV and

poliovirus. The hybrid virus is composed of poliovirus and HCV. The proteins

necessary for the proliferation and infection into other cells are produced by

poliovirus gene for the hybrid virus. The poliovirus gene is preceded by HCV

protease and the sites cleaved by the protease to construct this hybrid virus which

requires HCV protease NS3 activity for viability. This virus is composed,

sequentially, HCV NS3 protease domain plus its target site, and open reading

frame of poliovirus which is necessary for virus replication and virus particle

formation. The activity of the enzyme NS3 protease is necessary to survive the

hybrid virus.

The hybrid virus is proliferable in the cultivation system to settle the

conventional problem which HCV cannot be proliferated in vitro cultivation

systems. Therefore, the hybrid virus enables to develop anti-HCV drugs based

on protease inhibitor easily and precisely.

DETAILED DESCRIPTION OF THE DRAWINGS

The present invention will be described with reference to the

accompanying drawings, in which:

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FIG. 1 is schematic diagram of the genomic organization of poliovirus type 1 (Mahoney), NS3 △ C-PV1, and NS3 △ C'-PV1.

FIG. 2 is a photograph of plaque phenotypes of PV1 (M) and vNS3 △ C-PV1.

FIG. 3 is a photograph of RT-PCT analysis of vNS3 △ C-PV1 RNA.

Lane 1, 2 and 3 depict DNAs produced by PCR or RT-PCR using as template of pNS3 △ C-PV1, (+) sense RNA and (-) sense RNA, respectively.

FIGs. 4A, 4B and 4C are photographs of proteolytic processing patterns of PV1, NS3 △ C-PV1, and NS3 △ C'-PV1 polyproteins.

FIG. 5 is one-step graph curves of PV1 (M) and vNS3 △ C-PV1. HeLa cells were infected with either PV1 or vNS3 △ C-PV1 at a multiplicity of infection of 10.

FIG. 6A to FIG. 6E are shown the genetic sequence of pNS3 △ C-PV1 according to an embodiment of the present invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the following detailed description, only the preferred embodiment of the invention has been shown and described, simply by way of illustration of the best mode contemplated by the inventor(s) of carrying out the invention,   As will be realized, the invention is capable of modification in various obvious respects, all without departing from the invention,   Accordingly, the drawing and description are to be regarded as illustrative in nature, and not as restrictive.

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EXAMPLE

Material and Methods

Construction of plasmids

DNA manipulations were carried out by standard methods (Sambrook *et al.*, Molecular cloning: a laboratory manual (2nd ed.), Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989). The construction of the plasmids pTHE Δ 1219-1634 and pPNENPO are described elsewhere (Han *et al.*, *J. Gen. Virol.*, 76, 985-993, 1995; Alexander *et al.*, *Proc. Natl. Acad. Sci.*, USA, 91, 1406-1410, 1994). pUC19 was cleaved with XbaI, treated with Klenow, and digested with PstI. The resulting 2.6 kb fragment was then ligated to the 1.3 kb fragment of pTHEΔ1219-1634 treated with NcoI, Klenow, and PstI in succession. The result was the construct pUC19-NS3 Δ C-4 Δ C. pPNENPO and pUC19-NS3 Δ C-4 Δ C were digested with EcoRI or KpnI, respectively, and treated with Klenow enzyme. The linearized DNAs were ligated to result in the construct pPNENPO-NS3 Δ C-4 Δ C.

To construct pNS3 Δ C-4A-PV1, a polymerase chain reaction (PCR) was performed using the following four oligonucleotides:

Primer 1: 5'-TGATATCGAATTCCGG-3';

Primer 2: 5'-ATATGAGCTCCGCACTCTTCCATCTC-3';

Primer 3: 5'-ATATGAGCTCAGGTTTCATCACAGAAAG-3';

Primer 4: 5'-CTGTGCTAGCGCTTTTTG-3'.

The primers 1 plus 2 and pPNENPO-NS3 Δ C-4 Δ C were used to

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amplify the HCV protease NS3 △ C-4A region (PCR product 1). The PCR

product 1 contains a new SacI site right after the NS4A-coding sequence. The

primers 3 plus 4 and pPNENPO were used to amplify polioviral cDNA (746-2479)

with a new SacI site at the beginning of the poliovirus ORF (PCR product 2).

EcoRI and NheI digested pPNENPO, EcoRI and SacI digested PCR product 1,

and SacI and NheI digested PCR product 2 were ligated together to generate

plasmid pNS3 △ C-4A-PV1.

To construct pNS3 △ C-PV1, an oligonucleotide (5'-

AGCTCCGCAGCAGACGACGTCCTCACTGGCTTCCTCTTGCGCGCCTCCTCCT

CCGGTTGAGTTATCTGTGAAGAC-3') encoding the C-terminal end of NS5A (P1-

P10) and primer 1 were used to amplify the HCV region encoding amino acids

1016-1215. The PCR product was phosphorylated with polynucleotide kinase

and then digested with EcoRI. pNS3 △ C-4A-PV1 was cleaved with SacI,

treated with T4 DNA polymerase, and then digested with EcoRI. The larger

fragment of pNS3 △ C-4A-PV1 was ligated to the PCR product to generate pNS3

△ C-PV1. The junction sequences were confirmed by sequencing.

<u>Site-directed mutagenesis of the serine in the catalytic triad of the HCV</u>

<u>protease (Ser1165Ala).</u>

Site-directed mutagenesis was carried out by the Kunkel method as

previously described (Kunkel *et al.*, *Methods Enzymol.*, 154, 367, 1987).

pSK1016-1650 was generated by ligation of the DNA fragments isolated from

PvuII plus SalI-digested pTHE1016-1846 and SmaI plus SalI-digested pBluescript

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SK(+). E. coli strain RZ 1032 (ung⁻dut⁻) was transformed with pSK1016-1650, and

ssDNA was prepared from the transformant.   An oligonucleotide, 5'-

CTTGAAGGGCTCCGCG GGTGG-3', designed to change the serine residue in

the catalytic triad of HCV protease to alanine [Ser 1165(TCG) to Ala(GCG)] was

5   used for site directed mutagenesis.   The oligonucleotide was phosphorylated with

T4 polynucleotide kinase (Boehringer Mannheim) and annealed to the single

stranded pSK1016-1650 DNA, extended with sequenase (USB), and ligated with

T4 DNA ligase (Poscochem.) to result in covalently closed, circular, dsDNA.   The

ligated dsDNA was introduced into E. coli XL1-blue.   The base change was

10   confirmed by the presence of a newly generated SacII site and by sequencing.

pSK1016-1650 (Ser1165Ala) was used to construct pNS3 △ C'-PV1 by replacing

the XmaI fragment of pNS3 △ C-PV1 with the same fragment of pSK1016-1650

(Ser1165Ala).

In vitro transcription and translation

15   Plasmid DNAs were purified following the polyethylene glycol precipitation

method.   They were then linearized with appropriate restriction enzymes

downstream of the translation termination codon.   The linearized DNAs were

extracted with phenol/chloroform and ethanol-precipitated.   RNAs were

transcribed from the purified DNAs with T7 RNA polymerase (Boehringer

20   Mannheim) as described by the manufacturer.   These RNA transcripts were

translated in a rabbit reticulocyte lysate system (RRL) supplemented with 25%

HeLa cell lysate in the presence of [^{35}S] methionine.   The in vitro translation

reactions were carried out at 30 ℃ for 8 hours.   The [^{35}S]-labeled proteins were

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analyzed on 15% SDS-PAGE using the buffer system described by Nicklin et al. (1987). The intensity of the autoradiographic images was enhanced by fluorography using salicylic acid. Gels were dried and exposed to Kodak XAR-5 film or Agfa Curix RP1 film.

5   RNA transfection

An electroporation protocol was used to transfect HeLa cell monolayers with the viral RNAs prepared *in vitro*. Cells grown to 80% confluency were harvested. After two washes, the cells were resuspended in the medium at a concentration of 5×10^6 cells/ml. The cell suspension (1 ml) was mixed with 3

10   μg of RNA and placed in a 0.4 cm Gene Pulser cuvette (BIO-RAD). The cuvette was electrically pulsed at 360 volt and 960 μF using the Gene Pulser transfection apparatus (BIO-RAD). Then the cells were diluted with 9 ml of medium containing 10 % bovine calf serum, 5 % equine serum, and 5 % fetal calf serum.

         Plaque assay and one-step growth curve

15   Titers of virus stocks were measured by plaque assay on HeLa cell monolayers as follows. Cells were inoculated with virus, left for 30 minutes at room temperature, and then overlaid with Eagle medium containing 0.9 % Noble agar and 5 % fetal calf serum. Viral plaques were visualized with 0.5 % crystal violet solution 60 hours postinfection.

20   To measure one-step growth kinetics, virus was loaded onto 3.5 cm HeLa cell plates at a multiplicity of infection of 10 per cell and incubated at room temperature for 30 minutes to allow virus binding. Unbound particles were

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removed by two rounds of PBS washing. The infected cells were cultured at

37 °C in 2 ml of Eagle medium containing 10 % bovine calf serum and harvested

at the time points indicated in FIG. 5. The infectivity of the cell lysate at each

time point was titrated by plaque assay.

Reverse transcription PCR (RT-PCR)

Total RNA from virus-infected cells was isolated 4 hours postinfection as

described by Lu et al. (1995). cDNAs were synthesized using M-MuLV reverse

transcriptase and oligonucleotide primers corresponding to poliovirus nucleotides

556-570 and 789-806 (primer 5: 5'-GTGTTTCCTTTTATT-3'; primer 6: 5'-

GTGTTTCCTTTTATT-3'). Primer 5 and primer 6 were used in synthesizing

cDNAs from negative- and positive-sense RNA, respectively. Both of the primers

were used in the PCR reaction that followed. The PCR products were analyzed

on a 1.3 % agarose gel.

Labeling of newly synthesized proteins in virus-infected cells

HeLa cells were infected with poliovirus 1 (Mahoney: PV1) and vNS3 △

C-PV1 at a multiplicity of infection of 30 PFU per cell. The cells were incubated

in a medium lacking methionine for 10 minutes 3 hours after infection.

Subsequently, 100 μ Ci of [^{35}S] methionine was added to the medium and the

incubation continued for 40 minutes. The cells were then collected and lysed.

The lysate was analyzed on 15 % SDS-PAGE.

Immunoblot analysis

Virus-infected cells were lysed 4 hours postinfection. Total protein (13

μg) was resolved on a 15 % SDS-PAGE gel and transferred onto a nitrocellulose

membrane (Amersham). The blot was incubated overnight at 4 ˚C in blocking

solution [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5 % Tween 20, and 5 %

powdered skim milk] to block nonspecific binding. The primary antibody

5   (polyclonal antibody against HCV protease NS3) was added to the blocking

solution for 3 hours. The antibody was a generous gift from Dr. R.

Bartenschlager, Institute of Virology, Mainz, Germany. A horseradish

peroxidase-linked anti-rabbit IgG in a 1:10,000 dilution was used as the secondary

antibody.   Membrane-bound   antibodies   were   detected   by   enhanced

10   chemiluminescence reagents (ECL; Amersham).

Generation of a novel poliovirus containing HCV protease NS3 and its

target site

In order to develop an evaluation system for anti-HCV drugs targeting

protease NS3, a recombinant poliovirus that requires the activity of HCV protease

15   for its proliferation, was constructed. A schematic diagram of the genomic

structure of this virus is depicted in FIG. 1. The hatched and open boxes represent

HCV protease NS3 △ C and poliovirus polyprotein, respectively. The amino acid

sequence at the junction of HCV protease NS3 and poliovirus P1 is indicated by

single letter amino acid codes. The target site of HCV protease is marked by an

20   arrowhead. The mutated amino acid (S1165A) in NS3 △ C˙-PV1 is indicated by

an asterisk.

As shown in FIG. 1, in the case of polyprotein NS3 △ C-PV1, the protease

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domain of HCV NS3 is fused to the last 10 amino acids of NS5A (EEASEDVVCC), which are recognized by protease NS3. These amino acids residues are preceded by four glycines, alanine, and glutamine serving as a hinge region between the protease domain and the target site. It was expected that the HCV protease NS3 would cleave the peptide bond between cysteine at the C-terminus of the additional polypeptide and glycine at the N-terminus of poliovirus P1, since HCV protease NS3 is known to cleave between the cysteine or threonine at the P1-residue and the small uncharged residues at the P1'-residue (Kolykhalov et al., J. Virol. 68, 7525-7533, 1994; Komoda et al., J. Virol., 68, 7351-7357, 1994).

Upon transfection, the RNA transcript encoding NS3 △ C-PV1 produced a virus designated vNS3 △ C-PV1. On the other hand, the RNA transcript similar to NS3 △ C-PV1 but containing a serine to alanine mutation at the catalytic triad of HCV protease NS3 (NS3 △ C'-PV1) did not yield virus. This strongly suggests that HCV protease NS3 activity is essential for the viability of the hybrid virus vNS3 △ C-PV1. The virus vNS3 △ C-PV1 has a small plaque phenotype at 37 ℃ as shown in FIG. 2. (compare plaque sizes in panel PV1 with vNS3 △ C-PV1) or at 30 ℃ (data not shown).

The virus vNS3 △ C-PV1 is stable through at least five passages. The integrity of vNS3 △ C-PV1 was confirmed by analyzing the viral RNAs in cells inoculated with a virus stock of the fourth passage. Lanes 1, 2 and 3 depict DNAs produced by PCR or RT-PCR using as templates plasmid pNS3 △ C-PV1, (+) sense RNA, respectively. The inserted HCV sequence in the poliovirus

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genome was amplified by RT-PCT with primers annealing to the poliovirus 5'

nontranslated region and the P1-coding region. The viral RNAs of the fifth

passage retained the inserted HCV protease and NS5A sequences as shown in

FIG. 3. Both positive and negative sense viral RNAs of the expected sizes were

5   detected (FIG. 3, lanes 2 and 3, respectively). RT-PCR analysis of the genomic ·

RNAs isolated from vNS3 △ C-PV1 virion particles revealed the same RT-PCR

products as the RNAs isolated from HeLa cells (data not shown). The junction

sequence between HCV and poliovirus was confirmed by DNA-sequencing of the

PCR products and the sequences of hepatitis C surrogate virus genome is

10   presented in FIG. 6. This indicates that vNS3 △ C-PV1 is stable at least for five

passages.

<u>Proper proteolytic processing of vNS3 △ C-PV1 polyprotein requires HCV</u>

<u>NS3 protease activity.</u>

Proteolytic processing patterns of polyproteins encoded by poliovirus,

15   vNS3 △ C-PV1, and NS3 △ C˙-PV1 RNA were analyzed by *in vitro* transcription

and translation of pT7-PC1, pNS3 △ C-PV1, and pNS3 △ C˙-PV1. Most of the

polioviral proteins were detected as *in vitro* translation products of the PV1 RNA

transcript (FIG. 4A, lane 1). Two additional protein bands of 123 kDa and 26 kDa

were apparent among the translation products of NS3 △ C-PV1 as compared to

20   the PV1-translation product (FIG. 4A, lane 2). The 123 kDa and 26 kDa proteins

are most likely NS3 △ C-P1 fusion protein and the processed NS3 △ C,

respectively, since the cleavage of poliovirus at the P1/P2 junction occurs very

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fast, even during the process of translation. Translation of vNS3 △ C'-PV1 also

yielded the 123 kDa protein. But the 26 kDa and the P1 protein were not

detected among the translation products from this construct (FIG. 4A, lane 3).

This indicates that proteolytic cleavage of the HCV/poliovirus junction does not

5   occur in NS3 △ C'-PV1, and that the cleavage of this junction is mediated by the

protease activity of NS3. It also shows that the failure of virus production with the

NS3 △ C'-PV1 construct is most likely due to the lack of the P1 polypeptide which

is a protein processed into viral coat protein (VP0, VP1, and VP3). In other

words, HCV NS3 activity is essential for proliferation of the vNS3 △ C-PV1.

10   Interestingly, complete processing of the viral capsid proteins (VP0, VP1, and

VP3) from the NS3 △ C'-PV1 polyprotein was not detected. On the other hand,

the processed capsid proteins were apparent along with the precursor NS3 ∧ C-

P1 (compare lane 2 with lane 3 in FIG. 4A). The blockage of proteolytic

processing of the NS3 △ C'-PV1 polyprotein may be due to the lack of

15   myristoylation of the glycine residue at the N-terminus of P1 that has been shown

to be important in the processing of P1 by poliovirus protease 3C-3D (Kräusslich

et al., J. Virol., 64, 2433-2436, 1990).

The proteolytic processing was also investigated for PV1 and vNS3 △ C-

PV1 by labeling newly synthesized proteins with [^{35}S] methionine in virus-infected

20   cells. The labeling in vNS3 △ C-PV1-infected cells showed four additional bands

with apparent molecular weights of 123 kDa, 89 kDa, 63 kDa and 26 kDa as

indicated by arrow heads (FIG. 4B, compare lane 2 with lane 3). These proteins

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are most likely NS3 △ C-P1, NS3 △ C-VP0-VP3, NS3 △ C-VP0, and NS3 △ C,

respectively (FIG. 4B). This assumption is supported by the Western blotting

analysis using an antibody against HCV NS3 and HeLa cell extracts after infection

with either wild type poliovirus or vNS3 △ C-PV1 (FIG. 4C). Among the proteins

of vNS3 △ C-PV1-infected cells, the anti-NS3 antibody recognized the 63 kDa

and the 26 kDa polypeptides corresponding to vNS3 △ C-VP0 and NS3 △ C,

respectively (FIG. 4C, lane 3). A longer exposure of the X-ray film also revealed

the 123 kDa band and the 89 kDa band corresponding to NS3 △ C-P1 and NS3

△ C-VP0-VP3, respectively (data not shown). On the other hand, no bands were

detected in the Western analysis of cell extracts from wild type poliovirus- or

mock-infected cells (FIG. 4C, lanes 1 and 2). The results indicate that proteolytic

processing of the artificial polyprotein occurs as expected, even though the

HCV/poliovirus junction cleavage happens slower than the P1/P2 junction

cleavage. The relative intensities of the NS3-related bands varied depending on

the methodologies used to detect the polypeptides. For instance, a strong band

of NS3 △ C-P1 was detected by in vitro and in vivo labeling, but only a weak band

of NS3 △ C-P1 was detected by Western blot analysis. The [^{35}S] methionine

labeling method reveals only newly synthesized polypeptides and larger proteins,

containing more ·methionine residues, give stronger signals. Western blot

analysis, on the other hand, reveals all of the proteins interacting with the primary

antibody, and the intensity of the signal reflects the molarities of the proteins

rather than their size. Therefore, [^{35}S]methionine labeling methods are expected

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to give relatively stronger signals for precursor forms than Western blot analysis

methods.

One-step growth kinetics of chimeric virus vNS3 △ C-PV1

FIG. 5 was one step growth curve for vNS3 △ C-PV1. While maximum

production of wild type poliovirus was reached 4 hours postinfection (FIG. 5, open

squares), that of vNS3 △ C-PV1 was delayed by about 2 hours (FIG 5, closed

circles). In FIG. 5, the titer of vNS3 △ C-PV1 at the peak was about 1`.5 orders

of magnitude lower than that of wild type poliovirus, as viral titers at 9 hours

postinfection was compared. The small plaque phenotype of vNS3 △ C-PV1

may thus be a result of delayed virion particle formation and smaller burst size.

The HCV protease NS3 activity is necessary for noble poliovirus according

to the present invention to survive. This virus is useful for testing the efficacy of

anti-virus drugs which targets protease NS3.

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WHAT IS CLAIMED IS:

1.   A recombinant gene coding hepatitis C surrogate virus

comprising:

a virus gene coding picornavirus;

a protease gene coding hepatitis C virus protease NS3 which is in an

open reading frame (ORF) of said picornavirus; and

a target gene coding target sites of said hepatitis C virus protease NS3

which is in said open reading frame of said picornavirus.

2.   The recombinant gene coding hepatitis C surrogate virus of claim

1, wherein said picornavirus is poliovirus.

3.   The recombinant gene coding hepatitis C surrogate virus of claim

1, wherein said target site is selected from the group consisting of NS5A/5B,

NS4A/4B and NS4B/5A.

4.   The recombinant gene coding hepatitis C surrogate virus of claim

1, wherein said hepatitis C surrogate virus protease NS3 cleaves the peptide bond

between cysteine of P1 and glycine of P1' as a target site.

5.   A screening method of anti hepatitis C virus drugs comprising the

step of:

detecting a material which inhibits surrogate virus proliferation expressed

by said recombinant gene for coding hepatitis C surrogate virus of claim 1.

6.   A measuring method of anti-hepatitis C virus drugs activity

comprising the steps of:

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proliferating the surrogate virus expressed by said recombinant gene

coding hepatitis C surrogate virus of claim 1 in the presence of anti-hepatitis C
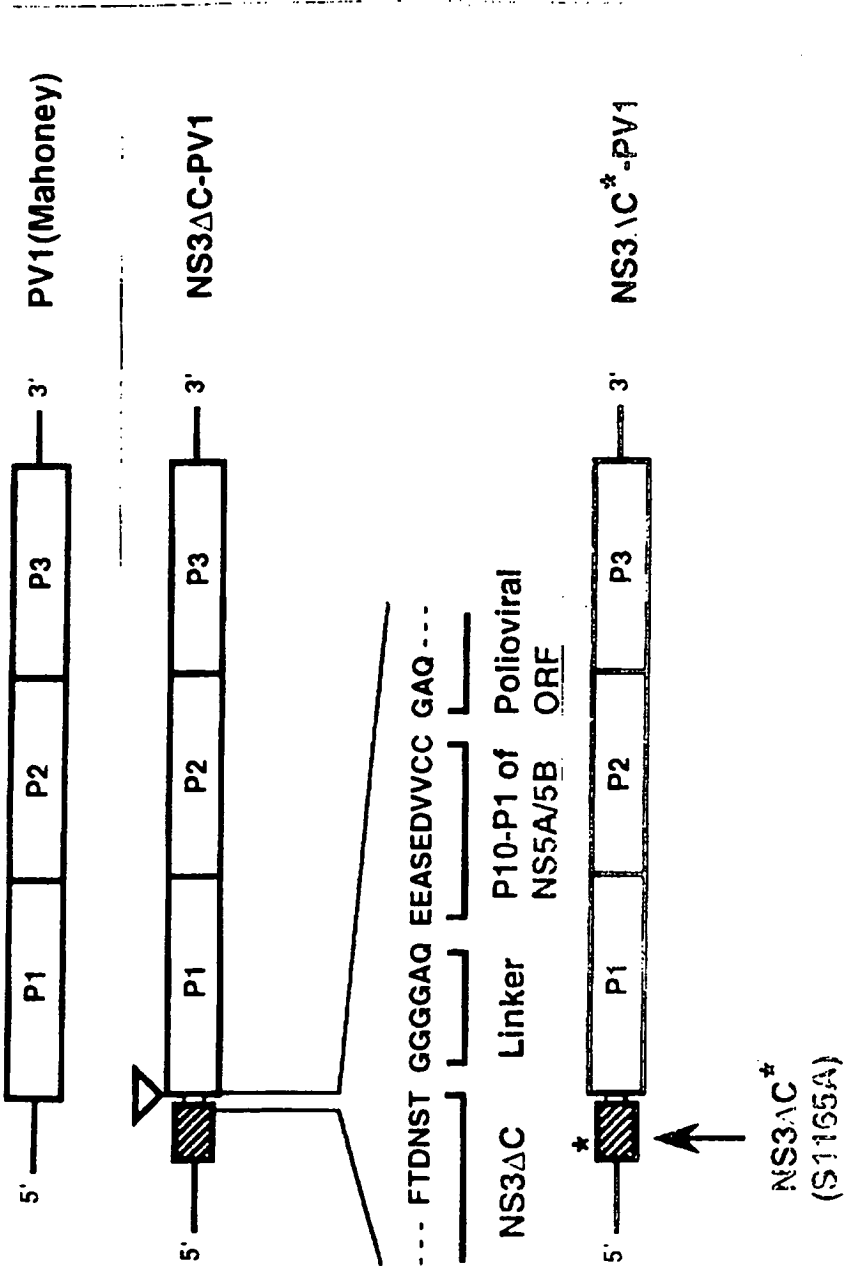
virus drugs; and

measuring the proliferating ability of said hepatitis C surrogate virus.
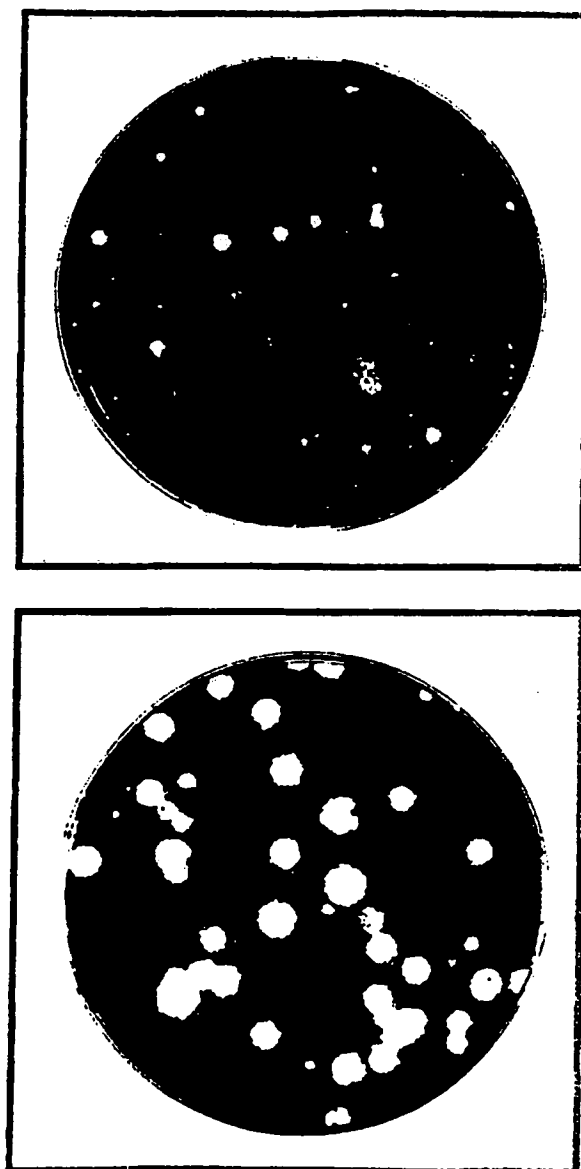
7.   A hepatitis C surrogate virus comprising:

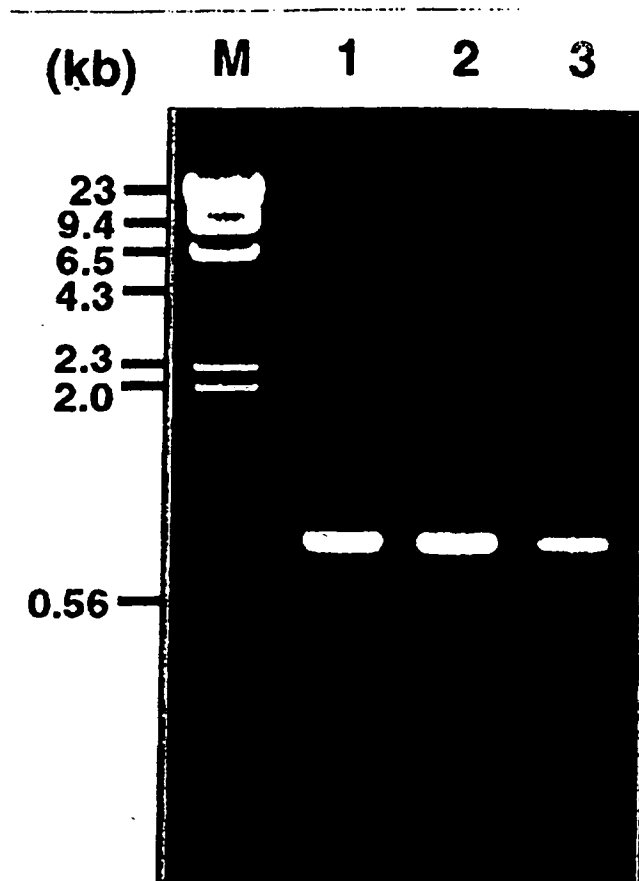a   recombinant gene for coding hepatitis C surrogate virus of claim 1.
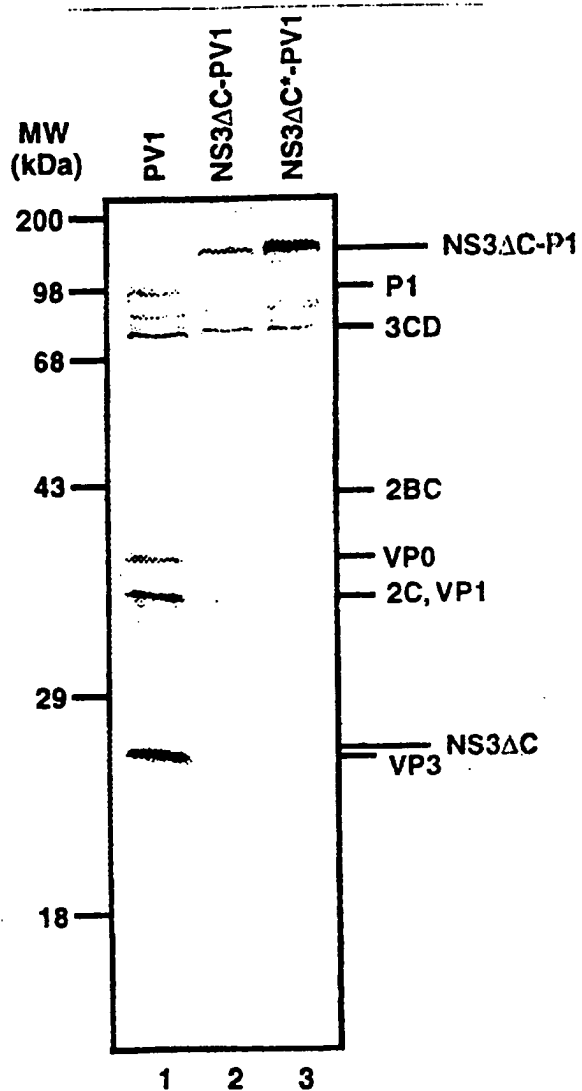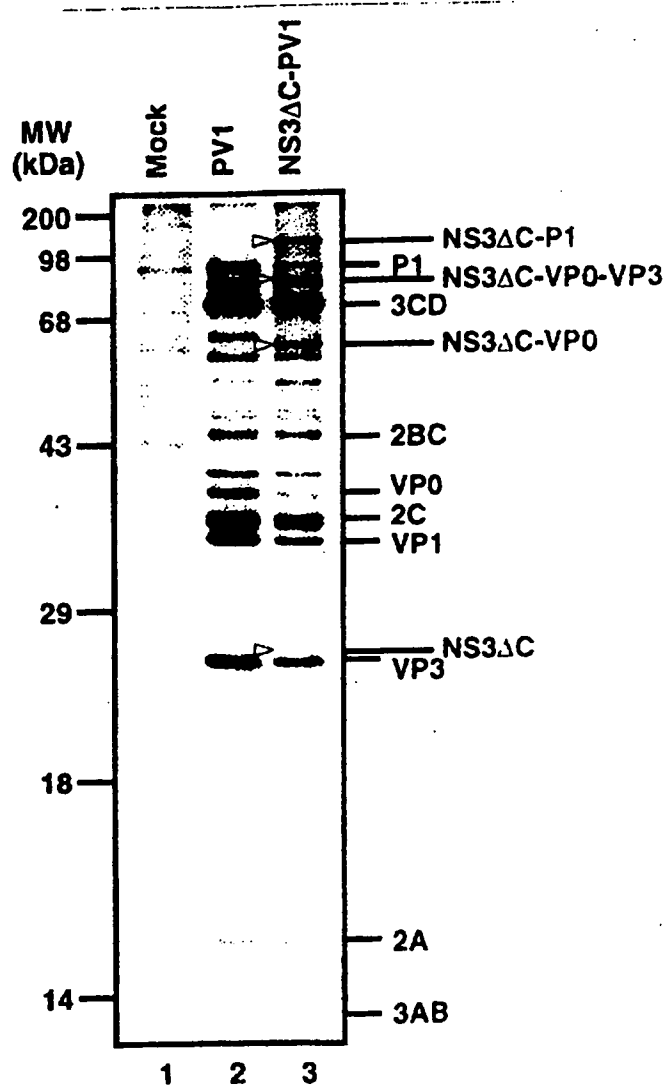
FIG. 1

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FIG. 2

vNS3△C-PV1

PV1

FIG. 3

FIG. 4A
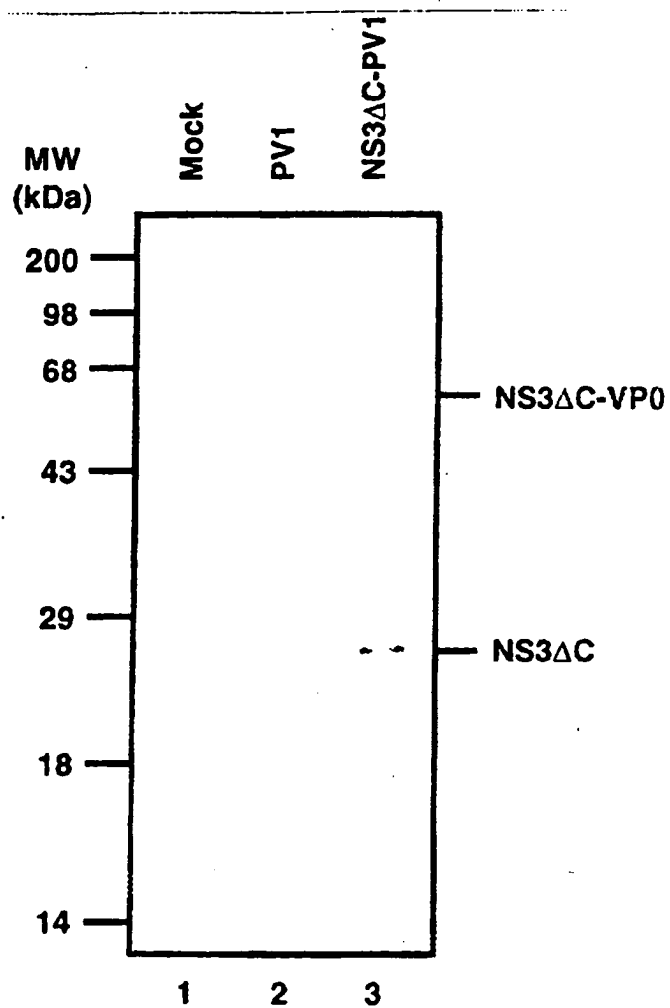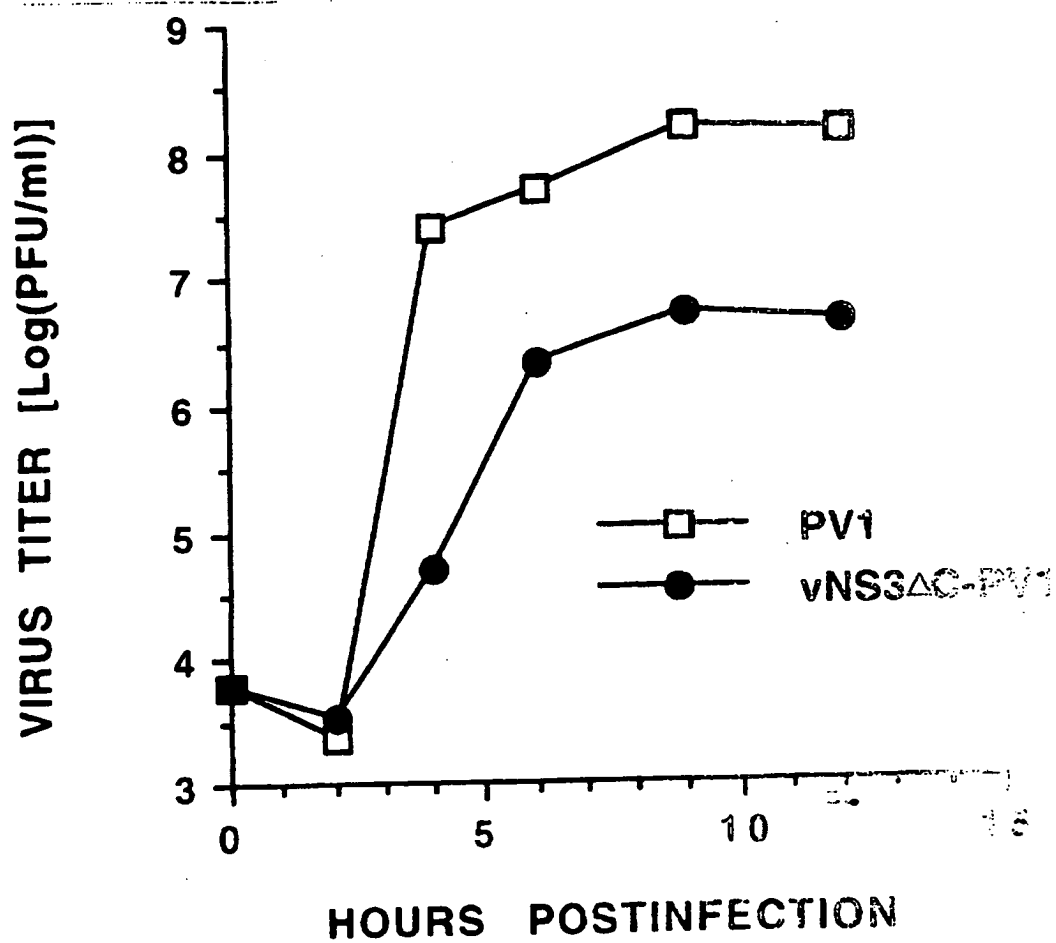
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FIG. 4B

FIG. 4C

FIG. 5

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FIG. 6A

```
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CTGTTTTATACTCCCTTCCCGTAACTTAGACGCACAAAACCAAGTTCAATAGAAGGGGG
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ACAAGCACTTCTGTTTCCCCGGTGATGTCGTATAGACTGCTTGCGTGGTTGAAAGCGACG
GATCCGTTATCCGCTTATGT
ACTTCGAGAAGCCCAGTACCACCTCGGAATCTTCGATGCGTTGCGCTCAGCACTCAACCC
CAGAGTGTAGCTTAGGCTGA
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GCTAACGCCATGGGACGCTA
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GCAGGTGGTCACAAACCAGTGATTGGCCTGTCGTAACGCGCAAGTCCGTGGCGGAACCGA
CTACTTTGGGTGTCCGTGTT
TCCTTTTATTTTATTGTGGCTGCTTATGGTGACAATCACAGATTGTTATCATAAAGCGA
AGACGGTATAGCTTGATATCG
AATTCCGGGGATCCTCTAGCATGGCCAGTCATCATCATCATCATCATGGAATTGTCGAC
AATTCCCCGGCCGATAGCCTT
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TACTTGGTTGCATCATCAC
TAGCCTCACAGGCCGGGACAAGAACCAAGTCGAGGGGGAGGTTCAAGTGGTTTCCACCGC
AACACAATCTTTCCTGGCGA
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CCTCTCCCAGACCAGTCTCCTACTTGAAGGGCTCCTCGGGTGGTCCACTGCTCTGCCCTTC
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GTCTCCGGTCTTCACAGATAACTCAACCGGAGGAGGAGGCGCGCAAGAGGAAGCCAGTGA
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CTCAGGTTTCATCACAGAAAGTGGGCGCACATGAAAACTCAAATAGAGCGTATGGTGGT
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CATCAAGGATGTCCTGATAAAAACAGCCCCAATGCTAAACTCGCCAAACATAGAGGCTT
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TGCAATTAACACTGGGAAACTCCACTATAACCACACAGGAGGCGGCTAATTCAGTAGTC
GCTTATGGGCGTTGGCCTGAA
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TGCAGGTTTTATACGCTAGA
CACCGTGTCTTGGACGAAAGAGTCGCGAGGGTGGTGGTGGAAGTTGCCTGATGCACTGAG
GGACATGGGACTCTTTGGGC
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GCCTCCAAATTCCACCAGGGG
GCACTAGGGGTATTCGCCGTACCAGAGATGTGTCTGGCCGGGGATAGCAACACCACTACC
ATGCACACCAGCTATCAAAA
TGCCAATCCTGGCGAGAAAGGAGGCACTTTCACGGGTACGTTCACTCCTGACAACAACCA
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TCTGCCCGGTGGATTACCTCCTTGGAAATGGCACGTTGTTGGGGAATGCCTTTGTGTTCC
CGCACCAGATAATAAACCTA
CGGACCAACAACTGTGCTACACTGGTACTCCCTTACGTGAACTCCCTCTCGATAGATAGT
ATGGTAAAGCACAATAATTG
GGGAATTGCAATATTACCATTGGCCCCATTAAATTTTGCTAGTGAGTCCTCCCCAGAGA
TTCCAATCACCTTGACCATAG
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FIG. 6B

CCCCTATGTGCTGTGAGTTCAATGGATTAAGAAACATCACCCTGCCACGCTTACAGGGCC
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CTCCAAGGAAATTCCGGCACTCACCGCAGTGGAAACTGGGGCCACAAATCCACTAGTCCC
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ACCGTGGATAACCCAGCTTCCACCACGAATAAGGATAAGCTTTTTGCAGTGTGGAAGAT
CACTTATAAAGATACTGTCCA
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GCTCCAGTGCCCGAAAAATGG
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CCAGCCCGGATCTCGGTACC
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AGGGTTGGTTGCATTTTCAGA
CATTAGAGACTTGTATGCCTACGAAGAAGAAGCCATGGAACAAGGCCTCACCAATTACA
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AGACACCACAACAGTGCTCGC

FIG. 6C

```
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GTCAGGAACACCAGGAAATTC
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TGAAGTGGATGAGTACATGAAAGAGGCAGTAGACCACTATGCTGGCCAGCTCATGTCAC
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FIG. 6D

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CACCACGATGCCTGCAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACT
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FIG. 6E

```
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CAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCG
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GCGGCCTTTTTACGGTTCCT
GGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGA
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AATGGTGCATGCAAGGAGAT
GGCGCCCAACAGTCCCCCGGCCACGGGGCCTGCCACCATACCCACGCCGAAACAAGCGCTC
ATGAGCCCGAAGTGGCGAG
CCCGATCTTCCCCATCGGTGATGTCGGCGATATAGGCGCCAGCAACCGCACCTGTGGCGCC
GGTGATGCCGGCCACGATG
CGTCCGGCGTAGAGGATCCCGCGAAATTAATACGACTCACTATAGG
```

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC^6: C 12 N 15/51; C 12 Q 1/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC^6: C 12 N 15/51; C 12 Q 1/70

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIL, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Patent Abstracts of Japan, Vol.16, No.419 (C-981), 1992, Kokai No. 4-144 686 (KUNITADA SHIMOTOONO), abstract.	1
A	WO 92/08 734 A1 (CHIRON CORPORATION) 29 May 1992 (29.05.92), claims 1-6.	1

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 September 1997 (01.09.97)	05 September 1997 (05.09.97)

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A-1014 Vienna
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Wolf

Telephone No. 1/53424/436 |

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
WO A1 9208734	29-05-92	AU A1 90267/91	11-06-92
		AU B2 668078	26-04-96
		CA AA 2095521	09-05-92
		CZ A3 9300824	13-04-94
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		EP A4 556292	18-01-95
		FI AO 932025	05-05-93
		FI A 932025	07-06-93
		FI A 971702	21-04-97
		FI AO 971702	21-04-97
		HU AO 9301336	28-10-93
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		JP T2 6504431	26-05-94
		NO AO 931680	07-05-93
		NO A 931680	28-06-93
		NO A 972213	14-05-97
		NO AO 972213	14-05-97
		PT A 99466	30-10-92